CHEMICAL STRATEGIES OF THE BEETLE *METOECUS PARADOXUS*, SOCIAL PARASITE OF THE WASP *VESPULA VULGARIS*

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Abstract - The parasitoid beetle *Metoecus paradoxus* frequently parasitizes colonies of the common wasp, *Vespula vulgaris*. It penetrates a host colony as a larva that attaches itself onto a foraging wasp's body, and once inside the nest, it feeds on a wasp larva inside a brood cell and then pupates there. When the beetle emerges, avoiding detection by its wasp host is crucial. Here, we test whether adult *M. paradoxus* beetles avoid detection by mimicking the cuticular hydrocarbon profile of their host. The beetles appear to be chemically adapted to their main host species, the common wasp, because they share significantly more hydrocarbon compounds than they do with the related German wasp, *V. germanica*. In addition, aggression tests showed that adult beetles were attacked less by common wasp workers than by German wasp workers. Our results further indicated that the host-specific compounds were, at least partially, produced through the recycling of the prey's hydrocarbons, and were not acquired through contact with the adult host. The chemical profile of the beetles moreover shows an overproduction of the wasp queen pheromone nonacosane (n-C_{29}), suggesting that beetles might mimic the queen's pheromonal bouquet.

Key Words - Chemical mimicry, Cuticular hydrocarbons, Social parasitism, Vespidae, *Vespula germanica*, Rhipiphoridae

INTRODUCTION

The chemical strategies that parasites employ to surpass the recognition system of their hosts offer a fascinating object of study within the field of chemical ecology (Bagnères and Lorenzi 2010, Dettner and Liepert 1994). It has been established that these strategies often involve mimicking host cuticular hydrocarbon (CHC) profiles, which have been demonstrated to play a key role in nestmate recognition (Akino et al. 2004, Dani et al. 2001, Gamboa 2004, Singer 1998, van Zweden et al. 2010, van Zweden and d'Ettorre 2010). Indeed, in many social insect species, colony members can distinguish nestmates from non-nestmates through comparison with their own colony odour template (known as their "Gestalt odour"), which often consists of a complex mix of different CHCs (Lenoir et al. 1999, Lorenzi et al. 1996, Singer 1998). These hydrocarbons are typically stored in the postpharyngeal gland (PPG), and can be exchanged between colony members through trophallaxis, grooming and physical contact, by which the colony Gestalt odour is spread among the colony members (Soroker et al. 1995). This fine-tuned recognition system enables social insects to keep most parasites out of the colony.

Nevertheless, as a counter strategy many parasites have evolved mechanisms to mimic the host's colony odour, a strategy that is commonly referred to as chemical mimicry (Howard et al. 1980, Howard et al. 1990, Vander Meer and Wojcik 1982). Generally, chemical mimicry is subdivided into two major types; namely chemical mimicry *sensu stricto* and chemical camouflage. The first is defined as a strategy in which the parasite actively biosynthesises host-specific compounds, while chemical camouflage is defined as a strategy in which the parasite acquires host cues passively through contact with the nest material (Bagnères and Lorenzi 2010, Howard and Blomquist 2005, Nash and Boomsma 2008). In reality, however, both mechanisms often occur simultaneously, making it difficult to distinguish one from the other (Bagnères and Lorenzi 2010). For example, caterpillars of the butterfly *Maculinea rebeli* use both strategies when parasitising *Myrmica schencki* ant colonies. They biosynthesise some host-specific brood pheromones and later acquire additional host-specific hydrocarbons through contact with adult workers (Akino et al. 1999). Additionally, parasites can employ an alternative strategy of chemical transparency, whereby they avoid detection by not synthesising the chemical compounds that are used by their host's recognition system,

thereby making them chemically invisible (Lenoir et al. 2001). An example of such chemical transparency is found in the wasp *Polistes atrimandibularis*, which parasitises *Polistes biglumis* colonies. It is thought that this social parasite succeeds in usurping *P. biglumis* host colonies by diluting recognition cues, as they have three to four times less CHCs than their host (Lorenzi and Bagnères 2002).

The subject of this study, the parasitic beetle *Metoecus paradoxus*, occurs mainly in nests of the eusocial wasp *Vespula vulgaris*. This beetle belongs to the subfamily Rhipiphorinae of which all species are obligatory parasites of various Hymenopteran taxa. Species belonging to the *Metoecus* genus specifically parasitise nests of eusocial Vespidae, and *M. paradoxus* is almost exclusively found in nests of the common wasp, *Vespula vulgaris*, although other host species have been reported (Heitmans and Peeters 1996, Spradbery 1973). In spring and summer, *V. vulgaris* workers collect decaying wood to build their nest, and it is at that time that the beetles' eggs, hidden in the crevices of the wood, hatch and the triungulinid larvae emerge. These larvae cling onto the foraging wasps' bodies and are carried into the nest where they enter a brood cell containing a fully grown wasp larva. Subsequently, the parasitoid consumes the entire wasp larva from the inside out and pupates inside the closed brood cell. After pupation, the beetle ecloses and chews through the brood cell cap, then leaving the colony to mate (Edwards 1980, Heitmans and Peeters 1996, Spradbery 1973). The exact period of time that the adult beetle remains inside the nest is as yet unknown, but adult fully pigmented beetles are frequently found inside wasp nests, which suggests that they remain there at least for one day after eclosing from their pupae. During this period that the adult beetle remains in the wasp nest, a crucial step in its survival is to avoid being attacked by its host.

Here, we hypothesised that *M. paradoxus* chemically mimics its host, the common wasp *Vespula vulgaris*, to avoid detection before leaving the colony to mate. We combined behavioural experiments and chemical analyses to determine the level of chemical host specificity to *V. vulgaris* as compared to its sister species *V. germanica*. Next, we conducted an isolation experiment to determine if beetles produce host-specific compounds themselves or acquire them through contact with the nest material after emerging from the brood cell. In addition, we used the fact that different wasp castes produce different hydrocarbon profiles to determine if the beetles might be recycling host-specific compounds from the prey item that they parasitised

on. Lastly, we compared CHC profiles of beetles with those of adult workers and queens to test the hypothesis that the beetles actually mimic queens the most and produce some key queen compounds to avoid attacks by the workers.

METHODS AND MATERIALS

Colony Collection. From July to October 2011, 83 *Vespula vulgaris* and 26 *V. germanica* nests were collected in the vicinity of Leuven, Belgium. After collection, the colonies were anaesthetized with carbon dioxide gas, and thoroughly checked for the presence of adult *Metoecus paradoxus* beetles (Figure 1). The nests were then placed in wooden boxes (15 × 17 × 40 cm) with a small hole (Ø = 4 cm), so that the workers could forage freely, and were placed outside in our apiary. The nests were checked every 3 to 4 days for the presence of adult beetles. These beetles randomly picked from colonies were used for aggression tests (see below). In addition, several combs were incubated and checked twice a day for newly emerged beetles, which were used for chemical analysis (see below).

Aggression Tests. In order to determine if the typical host, *V. vulgaris*, is less aggressive to the parasite beetles than its sister species, *V. germanica*, behavioural experiments were carried out to assess the degree of aggression towards introduced beetles. For these aggression tests, observation colonies of the two wasp species were set up, consisting of one comb and 200 to 300 workers, in an observation box (15.0 × 18.0 × 20.5 cm) with transparent walls and a copper wire to hold the comb in the middle of the box. A mirror was placed underneath the box so that the bottom of the comb could be observed. These observation colonies were placed under red light, so that it appeared dark for the wasps but they could still be observed. The experiments started approximately 30 minutes after the introduction of the wasps into the box (allowing time for the wasps to wake up after sedation and acclimatise to the new box). A total of eight beetles were each introduced into three different types of experimental colonies in a pseudo-random order: 1) their own *V. vulgaris* host colony, 2) a random alien *V. vulgaris* colony, and 3) a random alien *V. germanica* colony. Another two beetles were introduced only into a random alien *V. vulgaris* and a random alien *V. germanica* colony, because their host colonies were too weak to be used in experiments. The beetles were introduced on the comb through a tube in the top of the observation box. For 15 min, starting immediately after introduction, the aggressive interactions from workers to beetles were observed and counted. Five different types of behavioural interactions were observed: 1: antennation, 2: grabbing, 3: biting, 4: stinging attempts, and 5: dragging (sometimes accompanied by biting and/or stinging). The data were analysed using a

generalized linear mixed model (GLMM) with a binomial error structure with a logit link function. The number of aggressive (categories 2-5) and non-aggressive behaviours (category 1) were entered as dependent variables, and beetle individual, original beetle host colony and observation colony were included as random factors. Two models were run, one with species as a fixed factor (levels: *V. vulgaris* and *V. germanica*), in which only data of introductions into alien colonies were used, and one model with observation colony type (levels: own host colony, alien *V. vulgaris* colony, and alien *V. germanica* colony) as a fixed factor, in which only the data were used of the eight beetles that had been introduced into all three experimental colony types. Statistical analyses were carried out in R v3.1.0 (R Development Core Team) using the R package lme4 (Bates et al. 2012).

Cuticular Hydrocarbons, Comb Incubation, and Isolation Experiment. To assess the degree to which the cuticular hydrocarbon profiles of *M. paradoxus* individuals resemble those of their host *V. vulgaris* and of its sister species *V. germanica*, individuals of each of the species were obtained and washed for chemical analysis (see below). Firstly, we collected several beetles and wasps from their natural colony ($N_{collected}$ = 14 *M. paradoxus*, 38 *V. vulgaris*, and 10 *V. germanica*) and froze them at -20 °C for later analysis. This was done early in the season (July), at which point the wasp nests produce only workers, so that we could safely assume that all collected beetles were derived from worker brood cells. Secondly, to obtain beetles from male and queen brood cells, we placed combs of *V. vulgaris*, together with a dozen workers, in nest boxes in a climate room at 28 °C and 60-70 % relative humidity. Water and sugar water were provided *ad libitum*. As soon as pupal caps turned dark brown, meaning that the adult beetle was about to emerge, the cap was removed, the beetle taken out with clean forceps and frozen at -20 °C for later analysis ($N_{incubated}$ = 14 *M. paradoxus*). It was noted from which type of brood cell (male or queen) each of the beetles emerged to be able to compare their profiles with newly emerged *V. vulgaris* workers, males, and virgin queens. We used newly emerged wasps to make the ages more comparable. Newly emerged workers were collected from natural colonies and identified by their light pigmentation. Newly emerged males and queens were obtained through the same incubation procedure as the beetles ($N_{incubated}$ = 18 workers, 6 queens, 13 males).

Lastly, an isolation experiment was carried out to assess whether *M. paradoxus* beetles obtain their hydrocarbon profiles through active or passive contact with *V. vulgaris* workers. When a beetle was about to emerge from a worker cell ($N_{isolated}$ = 16), it was taken out from the cell with a forceps and placed by itself in a plastic box (20 × 9.5 × 6 cm) with a cardboard floor. They were kept under the same conditions in the climate room until they were frozen at -20 °C for later chemical analysis. We aimed to keep these isolated beetles alive for at least five days (mean ± SD = 7.81 ± 2.14 days), which is a relatively long isolation time considering that the average life expectancy of these beetles is only seven and eight days for females and males, respectively (Heitmans and Peeters 1996).

Chemical Analysis. Cuticular hydrocarbons were extracted by immersing entire individuals in 600 µL of HPLC-grade pentane (Acros Organics, Belgium) for the workers, males and beetles from small brood cells, while 1 mL of pentane was used for the queens and beetles from large (queen) brood cells (Figure 1). The first and the last 15 seconds of the 10 minute extraction, the samples were vortexed at 800 rpm. Thereafter, the solvent was allowed to evaporate at room temperature in a laminar flow cabinet. The extracts were subsequently resuspended in 50 µL of pentane for workers, males and beetles from small brood cells and in 100 µL of pentane for queens and beetles from large brood cells. Two microliters of this suspension were injected in an Agilent 6850 gas chromatograph (Agilent Technologies, US), equipped with a HP-1 capillary column (30 m × 320 µm × 25 µm), a splitless injector at 280 °C, a flame ionization detector (FID), and a helium carrier gas flow at 1.1 mL min^{-1}. The GC oven program had an initial hold at 70 °C for 1 min, after which the temperature was increased to 150 °C at a rate of 20 °C min^{-1}, and then to 320 °C at 3.5 °C min^{-1}. As a final step, the temperature was held at 320 °C for 5 min. Compounds were identified using an Agilent 7890A GC equipped with a ZB-5HT capillary column (30 m × 320 µm × 25 µm) coupled to 5975C Inert XL EI/CI mass spectrometric detector with electron ionization (70 eV), using the same temperature program as above. The transfer line was set to 280 °C and the helium carrier gas flow at 1.5 mL min^{-1}.

The peak areas of identified compounds were quantified using Agilent ChemStation (v. A.09.01, Agilent Technologies) and normalised to relative concentrations using a log-ratio transformation (Aitchison 1986) and scaled to unit variance. Retention indices of identified compounds were calculated by a cubic spline

interpolation function (Halang et al. 1978) using an Excel macro (available from the authors on request), as this provides estimates with better reproducibility across different instruments and temperature programs (Messadi et al. 1990). The data were analysed using principal component analysis (PCA) and multiple analysis of variance (MANOVA). Because we noticed that the *M. paradoxus* individuals had much higher relative proportions of linear alkanes and some alkenes on their cuticle than the *Vespula vulgaris* individuals (Figure 2; Table 2), the data were analysed for all compounds together and separately for the linear alkanes, methylalkanes, and alkenes. For each of these compound groups the raw data were again log-ratio transformed and scaled to ascertain independence of the other compound groups. All statistical analyses were carried out using R v3.1.0 (R Development Core Team 2011).

RESULTS

Collection. A total of 154 *Metoecus paradoxus* beetles (143 adults and 11 pupae) were collected from the *Vespula vulgaris* nests. There were no beetles found in any of the 26 *V. germanica* nests. The common wasp colonies contained between 0 and 11 beetles, with an average of 1.73 ± 2.02 (mean ± SD) per colony, and 66.3 % of all colonies contained at least one beetle. We found that the sex ratio of the adult beetles was biased towards females, as we collected approximately twice as many females (N = 95) as males (N = 48).

Host Specificity: Aggression Tests. Workers of *V. vulgaris* from a colony alien to the beetles were less aggressive towards the beetles than alien *V. germanica* workers were (binomial GLMM with species as fixed factor, $z = 2.017$, $p = 0.044$). When, however, observation colony type was used a fixed explanatory factor (levels: own *V. vulgaris* host colony, alien *V. vulgaris* colony, or alien *V. germanica*) there was no significant difference in the level of aggression between *V. vulgaris* workers of the beetles' own host colony and *V. vulgaris* workers from an alien colony (binomial GLMM with observation colony type as fixed factor, $z = 1.423$, $p = 0.155$), although the aggression was slightly higher in the alien *V. vulgaris* colonies (Figure 1). On the other hand, *V. germanica* workers were clearly more aggressive to the introduced beetles than were *V. vulgaris* of their own host colony (Figure 1; binomial GLMM with observation colony type as fixed factor, $z = 2.565$, $p = 0.010$).

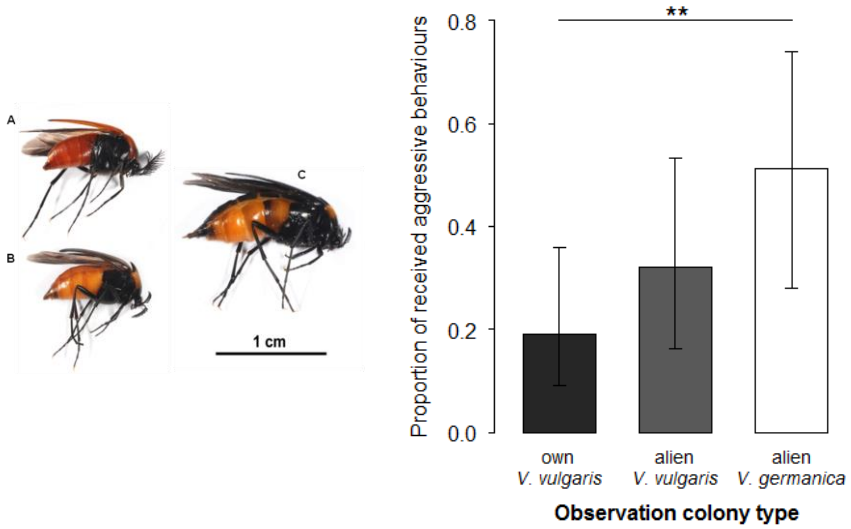

Fig. 1 Male *Metoecus paradoxus* (A) can be easily recognized by their darker orange colour and their double-branched antennae, when compared to females (B) with single-branched antennae, a lighter colour, and an ovipositor. Note the difference in size between beetles that developed in large queen cells (C) and beetles that developed in small worker brood cells. (D) The beetles received significantly less aggression when introduced into colonies of their most common host, *Vespula vulgaris*, compared to colonies of the sister species *V. germanica*. Colonies of *V. vulgaris* from which the beetles did not originate ("alien") treated the beetles slightly more aggressively than "own" colonies, but this effect was not significant. Columns depict estimated means and error bars 95 % confidence intervals; ** p = 0.010.

Host Specificity: Resemblance in CHC Profiles. In total, we identified 67 gas-chromatographic peaks in the beetles and the two wasp species (Figure 2; Table 1). Of the 56 typical *M. paradoxus* compounds, 51 (91.1 %) were also found in the *V. vulgaris* samples. The five additional beetle-specific compounds, namely 4 alkenes and 1 alkadiene, co-eluted in peaks 2, 9, 22, 28, and 37 (Table 1). In *V. vulgaris*, all of these peaks otherwise contained only a 3-methyl- or 4-methylalkane (Table 1). These peaks were regarded as methylalkanes in further quantitative analyses, since the alkenes/alkadienes were low in relative concentration and this makes the comparison with the wasps more appropriate since the two species share the methylalkanes. In contrast, only 34 of the 56 (60.7 %) typical *M. paradoxus* compounds could also be found in the *V. germanica* worker samples, so the qualitative resemblance with *V. vulgaris* was greater.

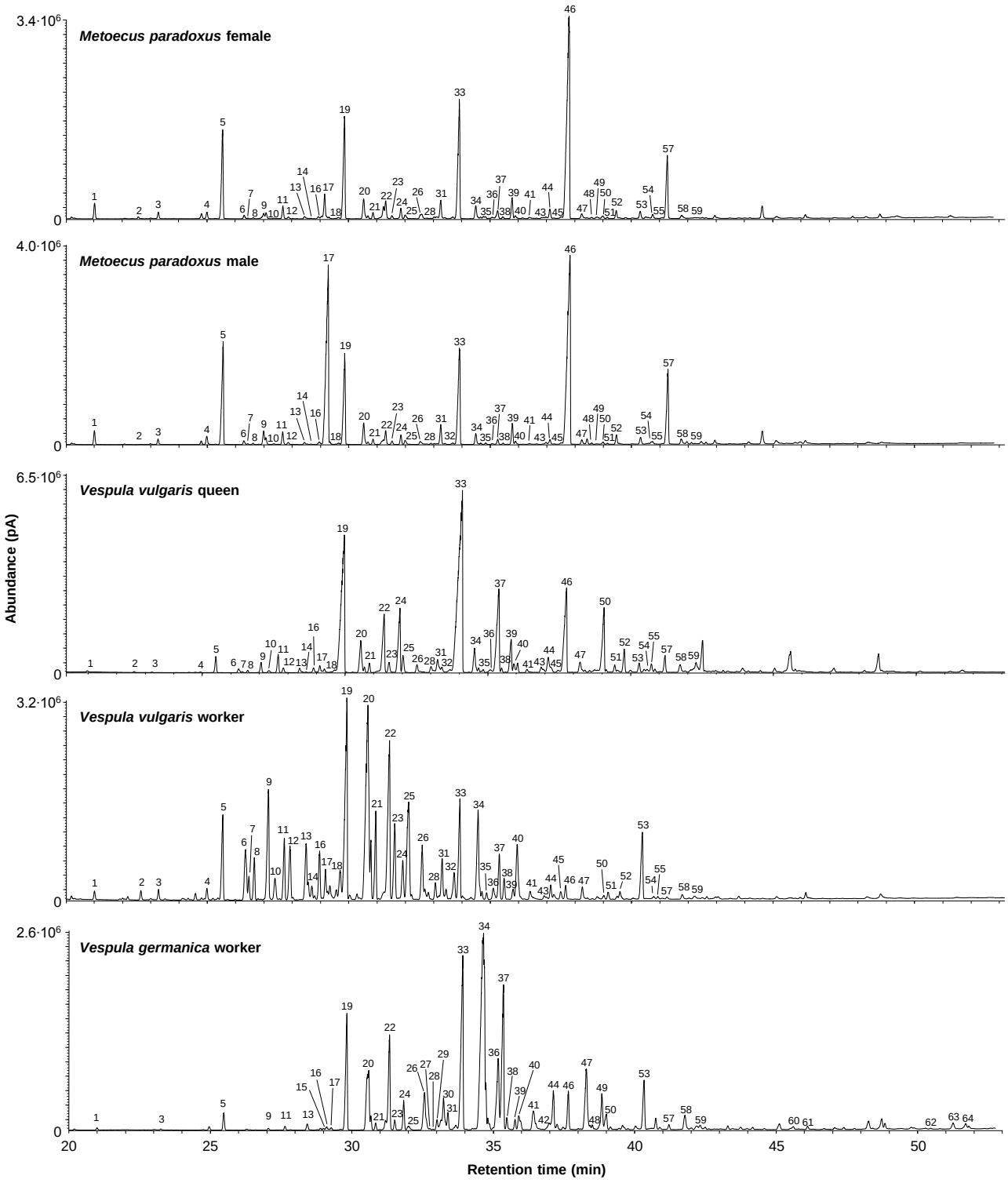

Fig. 2 Typical chromatograms of the different types of individuals: *Metoecus paradoxus* females and males, *Vespula vulgaris* queens and workers, and *V. germanica* workers. Labels refer to identification in Table 1.

TABLE 1 OVERVIEW OF IDENTIFIED CHCS FOR THE THREE SPECIES

Identified compounds in the species *Metoecus paradoxus*, *Vespula vulgaris* and *V. germanica*, with their label number in Figure 2, retention index, and average percentage in the different species. * The alk(adi)enes in these peaks were only found in the *M. paradoxus* samples.

Peak Nr.	Identified compound	Retention Index	*M. paradoxus* female	*M. paradoxus* female - isolated	*M. paradoxus* male	*M. paradoxus* male - isolated	*V. vulgaris* queen	*V. vulgaris* worker	*V. germanica* worker
1	n-C_{21}	2100	0.71	0.48	1.04	0.93	0.09	0.35	0.13
2	3-MeC_{21} + $C_{22:1}$ *	2173	0.22	0.41	0.07	0.01	0.05	0.22	-
3	n-C_{22}	2200	0.66	1.21	0.29	0.21	0.11	0.27	0.13
4	$C_{23:1}$	2275	0.83	1.74	0.37	0.19	0.16	0.34	-
5	n-C_{23}	2300	8.79	13.79	8.65	10.15	0.76	3.05	0.79
6	7 + 9 + 11-MeC_{23}	2337	0.40	0.21	0.45	0.34	0.20	2.22	-
8	5-MeC_{23}	2351	0.09	0.02	0.08	0.05	0.09	0.88	-
9	3-MeC_{23} + $C_{24:1}$ *	2374	1.53	1.84	1.80	1.30	0.43	2.72	0.35
10	5,y-diMeC23	2385	0.07	0.03	0.07	0.05	0.09	0.66	-
11	n-C_{24}	2400	1.54	3.07	0.88	1.35	0.99	1.86	0.46
12	3,y-diMeC23	2409	0.19	0.12	0.14	0.10	0.21	1.43	-
13	10 + 12 + 14-MeC_{24}	2434	0.32	0.14	0.24	0.17	0.28	1.98	0.33
14	6-MeC_{24}	2443	0.08	0.03	0.08	0.19	0.07	0.47	-
15	5-MeC_{24}	2451	-	-	-	-	-	-	0.03
16	4-MeC_{24}	2455	0.41	0.29	0.25	0.37	0.24	1.25	0.29
17	$C_{25:1}$	2465	3.94	5.38	31.33	29.59	0.53	1.58	0.37
18	4,y-diMeC24	2489	0.16	0.10	0.14	0.17	0.16	1.00	-
19	n-C_{25}	2500	8.51	8.09	6.95	13.27	16.14	11.92	6.98
20	7 + 9 + 11 + 13-MeC_{25}	2537	2.35	0.85	2.07	1.31	2.55	12.20	8.30
21	5-MeC_{25}	2551	0.48	0.15	0.35	0.19	0.54	2.46	0.38
22	3-MeC_{25} + $C_{26:1}$ *	2576	4.46	5.51	1.79	1.96	3.43	7.28	6.82
23	5,9-diMeC_{25}	2586	0.33	0.16	0.23	0.17	0.56	2.49	1.51
24	n-C_{26}	2600	0.95	1.19	0.48	0.46	4.43	1.82	1.37
25	3,11-diMeC_{25}	2610	0.48	0.29	0.31	0.23	0.96	4.21	0.69
26	8 + 10 + 12-MeC_{26}	2634	0.74	0.42	0.36	0.31	0.59	2.16	3.08
27	6-MeC_{26}	2644	-	-	-	-	-	-	0.41
28	4-MeC_{26} + $C_{27:2}$ *	2657	0.39	0.37	0.25	0.35	0.52	0.89	0.08
29	10,y-diMeC_{26}	2656	-	-	-	-	-	-	0.54
30	3-MeC_{26}	2667	-	-	-	-	-	-	1.90
31	$C_{27:1}$	2669	6.16	10.40	3.10	4.74	1.42	2.11	0.64
32	4,y-diMeC26	2690	0.18	0.10	0.12	0.11	0.24	1.13	-
33	n-C_{27}	2700	10.19	6.88	6.87	5.87	28.48	5.47	8.35
34	7 + 9 + 11 + 13-MeC_{27}	2733	1.47	0.56	0.93	0.53	2.47	6.22	22.40
35	5-MeC_{27}	2749	0.24	0.15	0.12	0.08	0.31	0.51	-
36	11,15 + 9,13 + 7,11-diMeC_{27}	2762	0.21	0.17	0.09	0.08	0.35	0.86	6.90
37	3-MeC_{27} + $C_{28:1}$ *	2774	1.59	2.08	0.38	0.39	7.40	2.97	8.22
38	5,15-diMeC_{27}	2783	0.14	0.07	0.08	0.05	0.35	1.03	1.15
39	n-C_{28}	2800	1.62	1.35	1.13	0.85	2.06	0.50	0.47
40	3,11-diMeC_{27}	2808	0.33	0.16	0.19	0.12	1.21	3.09	1.26
41	10 + 12 + 14-MeC_{28}	2833	0.19	0.11	0.10	0.05	0.31	0.71	1.70
42	9,y + 10,y-diMeC_{28}	2865	-	-	-	-	-	-	0.49
43	4-MeC_{28}	2860	0.30	0.25	0.13	0.08	0.57	0.26	-
44	$C_{29:1}$	2872	2.80	4.96	0.52	0.53	1.34	0.98	1.35
45	4,y-diMeC28	2891	0.17	0.10	0.12	0.11	0.20	0.45	-
46	n-C_{29}	2900	24.65	16.64	20.15	15.80	6.35	1.18	1.04
47	7 + 9 + 11 + 13-MeC_{29}	2930	1.07	0.79	0.55	0.32	1.48	1.51	3.21
48	5-MeC_{29}	2946	-	-	-	-	-	-	0.43
49	13,17 + 11,15 + 9,13-diMeC_{29}	2964	-	-	-	-	-	-	1.93
50	3-MeC_{29}	2969	0.77	1.21	0.14	0.11	4.51	0.35	0.90
51	5,9-diMeC_{29}	2977	0.10	0.05	0.06	0.04	0.18	0.52	-
52	n-C_{30}	3000	0.74	0.58	0.53	0.39	0.59	0.71	-
53	10 + 11 + 12 + 13 + 14-MeC_{30}	3046	1.01	1.13	0.52	1.83	1.35	2.05	2.03
54	x,y-diMeC_{30}	3054	0.49	0.77	0.13	0.12	0.15	0.13	-
55	$C_{31:1}$	3071	1.11	1.88	0.28	0.19	0.36	0.31	-
56	4-MeC_{30}	3090	0.06	0.05	0.05	0.06	0.22	0.11	-
57	n-C_{31}	3100	4.88	3.02	4.55	3.73	0.44	0.21	0.33
58	11 + 13 + 15-MeC_{31}	3134	0.49	0.26	0.30	0.20	0.69	0.41	0.56
59	11,17 + 13,17 + 15,19-diMeC_{31}	3162	0.41	0.41	0.21	0.21	2.75	0.49	0.21
60	11 + 13 + 15-MeC_{33}	3370	-	-	-	-	-	-	0.14
61	11,21-diMeC_{33}	3403	-	-	-	-	-	-	0.19
62	11 + 13 + 15 + 17-MeC_{35}	3693	-	-	-	-	-	-	0.09
63	11,21-diMeC_{35}	3766	-	-	-	-	-	-	0.27
64	7,11-diMeC_{35}	3801	-	-	-	-	-	-	0.27
65	11 + 12 + 13 + 17-MeC_{37}	4000	-	-	-	-	-	-	0.19
66	13,23-diMeC_{37}	4011	-	-	-	-	-	-	0.18
67	11,21-diMeC_{37}	4040	-	-	-	-	-	-	0.14

From a quantitative point of view, *M. paradoxus* also resembled *V. vulgaris* more than *V. germanica* (Figure 3; Table 1), although mimicry of the host's CHC profile was not perfect. In a PCA, based on the 34 CHC variables shared by the three species and only individuals from natural colonies, the common wasp workers (N = 31) plotted significantly closer on PC1 to the beetles (N = 14) than the German wasps (N = 10) (Figure 3; *M. paradoxus*: -5.84 ± 0.31 (estimate ± S.E.), *V. vulgaris*: 1.04 ± 0.17, *V. germanica*: 4.96 ± 0.35; *V. vulgaris vs. M. paradoxus*, $t = -22.08$, $p < 0.001$; *V. vulgaris vs. V. germanica*, $t = 11.17$, $p < 0.001$). As expected based on the chromatograms in Figure 2, compounds that were found to be more specific to *M. paradoxus* than to wasps were the linear alkanes and alkenes, which all loaded strongly negatively on PC1 (Table S1). When, however, the PCA was performed with only the 21 methylalkane variables (Figure 3), *M. paradoxus* and *V. vulgaris* plot together on the one side and *V. germanica* on the other, showing that the parasite and *V. vulgaris* are rather similar in their methylalkane profile, and that the chemical difference between the parasite and its host lies mostly in the linear alkanes and alkenes. Indeed, when using only linear alkanes in the PCA, the largest variation is that between beetles on the one side and the two wasp species on the other (Figure 3). This was mostly due to n-C_{25} and n-C_{26} being relatively more characteristic for the wasps, whereas especially n-C_{29} was more characteristic for the beetles (Table S1). In the alkene-only analysis, the predominant differentiation was that between male beetles and the rest, due to male beetles having a high relative concentration of pentacosene (x-$C_{25:1}$) on their cuticles (Table S1), whereas female beetles and wasps were more characterised by nonacosene (x-$C_{29:1}$) and hentriacontene (x-$C_{31:1}$).

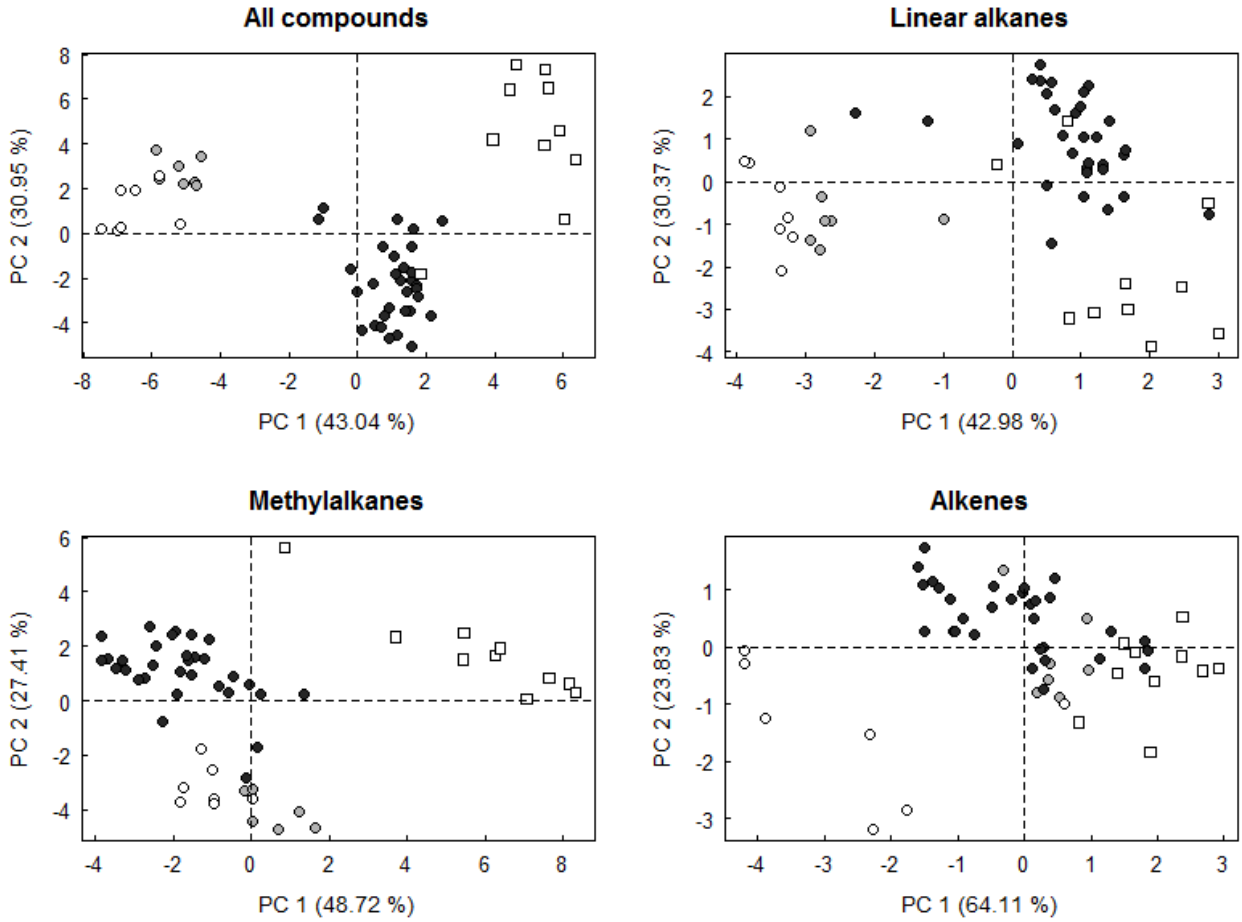

Fig. 3 PCA plots of CHC data of *Metoecus paradoxus* individuals, *Vespula vulgaris* workers, and *V. germanica* workers, all collected from natural nests. Panels from left to right: based on identified cuticular compounds shared by all three species (34 variables), based only on the linear alkane subset (9 variables), based only on methylalkane subset (21 variables), based only on alkene subset (4 variables). Grey circles = *M. paradoxus* females, white circles = *M. paradoxus* males, black circles = *V. vulgaris* workers, white squares = *V. germanica* workers.

Isolation Experiment. A PCA of the CHC data of the beetles (N = 14) and *V. vulgaris* workers (N = 31) collected from natural colonies (i.e. non-isolated), and the isolated beetles (N = 16), showed that the largest variation was that between beetles and wasps (Figure 4; Table 1). The major cause of this difference was again that beetles were generally characterised by a higher proportion of linear alkanes and alkenes in their profile, while wasp profiles had higher relative abundances of methylalkanes (Table S1; Table 1). The CHC

profiles of isolated beetles were significantly further away from the wasps than those of non-isolated beetles (GLM: estimate PC1 score ± SE: isolated: -6.26 ± 0.55, non-isolated: -3.83 ± 0.40, workers: 8.78 ± 0.48; isolated *vs.* non-isolated, $t = -4.43$, $p < 0.001$; workers *vs.* non-isolated, $t = 18.15$, $p < 0.001$). However, what stands out immediately in Figure 4 is that this isolation effect was only apparent in the female beetles (GLM: *M. paradoxus* individuals only, PC1 score ~ isolation × sex, isolation: $F_{1,26} = 41.45$, $p < 0.001$, sex: $F_{1,26} = 22.51$, $p < 0.001$, isolation × sex: $F_{1,26} = 9.49$, $p < 0.01$). When compound groups were analysed separately, a very similar pattern was found for the methylalkanes but not for the linear alkanes, where isolated and non-isolated beetles did not differ in their PC1 score (isolated: -2.24 ± 0.29, non-isolated: -2.50 ± 0.21, isolated *vs.* non-isolated, $t = 0.91$, $p = 0.368$), nor for the alkenes, where again it were mostly the male beetles that separated from the rest of the individuals (Figure 4; Table S1).

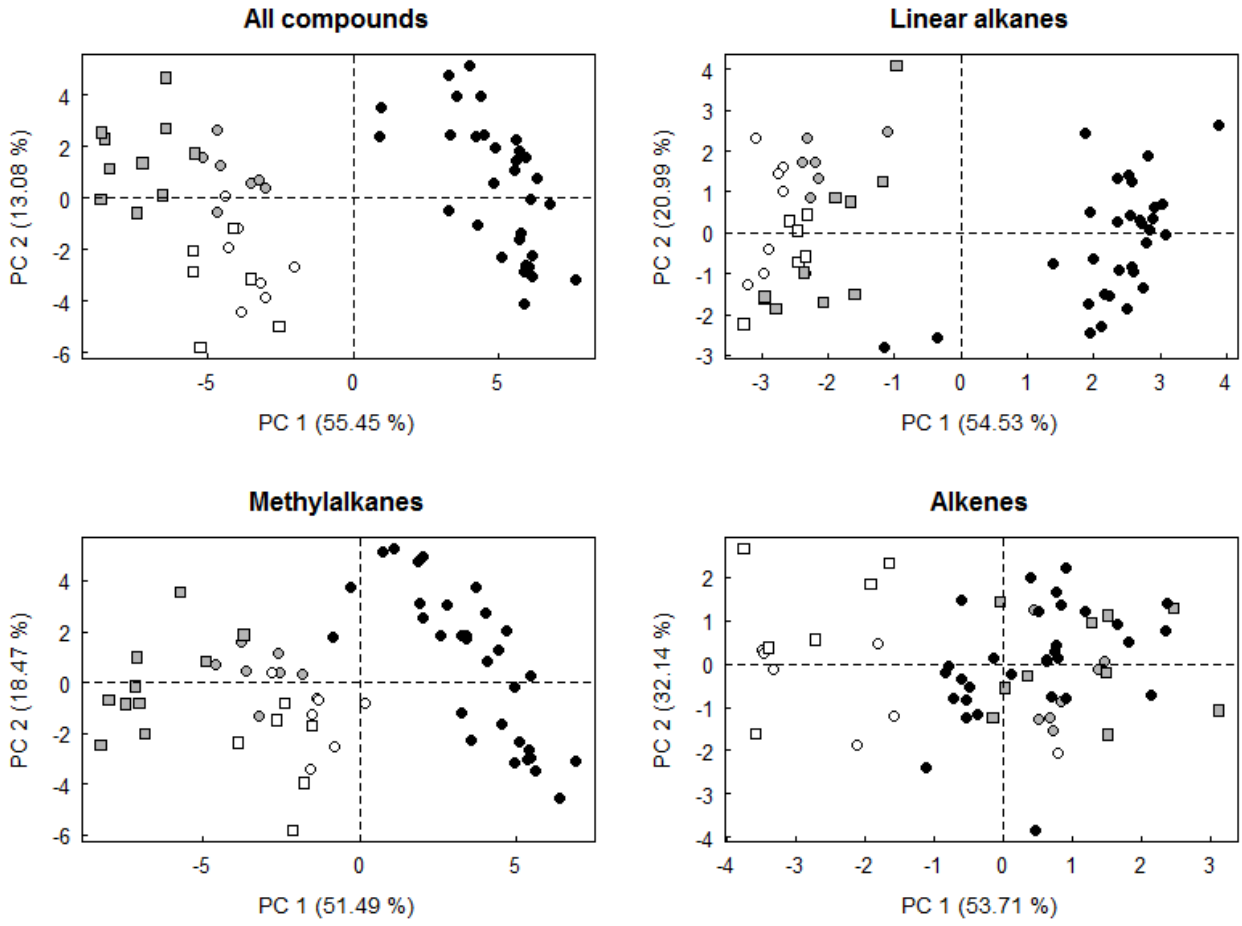

Fig. 4 PCA plots of CHC data of isolated and non-isolated *M. paradoxus*, and *V. vulgaris* workers. Panels from left to right: based on all identified cuticular compounds (51 variables), based only on the linear alkanes (11 variables), based only on methylalkanes (35 variables), based only on alkenes (5 variables). Grey circles = non-isolated *M. paradoxus* females, white circles = non-isolated *M. paradoxus* males, grey squares = isolated *M. paradoxus* females, white squares = isolated *M. paradoxus* males, black circles = *V. vulgaris* workers.

Hydrocarbon Recycling. The analysis on the CHC data of beetles from different brood cell types (N = 28), i.e. that fed on and developed in the brood cells of larvae belonging to different castes (workers, queens, and males), and the data of newly emerged wasps belonging to different castes (N = 37), showed that, apart from the larger pattern of separation between the parasite and its host, there was a more fine-scaled pattern that separated between individuals originating from different types of brood cells (Figure S1; Table 2).

TABLE 2 MANOVA ANALYSES OF CHCS ACCORDING TO SPECIES AND BROOD CEL TYPE

Results of MANOVAs using the first two PCs as dependent variables, and species and brood cell type as explanatory variables (see also Figure 4). The original PCAs have been performed either using all compounds, linear alkanes only, methylalkanes only, or alkenes only. df = degrees of freedom, num df = numerator degrees of freedom, den df = denominator degrees of freedom, *** = significance at 0.001-level.

All compounds	df	Wilks' λ	approx. F	num df	den df	p
Species	1	0.070	396.94	2	60	< 0.001 ***
Brood cell type	2	0.692	6.06	4	120	< 0.001 ***
Residuals	61					

Linear alkanes	df	Wilks' λ	approx. F	num df	den df	p
Species	1	0.207	114.60	2	60	< 0.001 ***
Brood cell type	2	0.858	2.39	4	120	0.055
Residuals	61					

Methylalkanes	df	Wilks' λ	approx. F	num df	den df	p
Species	1	0.149	171.81	2	60	< 0.001 ***
Brood cell type	2	0.594	8.94	4	120	< 0.001 ***
Residuals	61					

Alkenes	df	Wilks' λ	approx. F	num df	den df	p
Species	1	0.284	75.50	2	60	< 0.001 ***
Brood cell type	2	0.855	2.44	4	120	0.051
Residuals	61					

DISCUSSION

Our study demonstrates that the parasitic beetle *Metoecus paradoxus* frequently parasitizes *Vespula vulgaris* colonies, with 66.3 % of all collected colonies containing one or more beetles. By contrast, in *V. germanica* colonies, no beetles were detected. Hence, even though the beetle has occasionally been reported in *V. germanica* nests (Carl and Wagner 1982, Heitmans and Peeters 1996), it appears that *M. paradoxus* has *V. vulgaris* as its main host. Our chemical analyses showed that the cuticular hydrocarbon (CHC) profiles of the beetles resembled the profiles of the common wasp more than those of the congeneric German wasp, although mimicry was not perfect. In particular, we found that the beetles share 51 hydrocarbon compounds with their common wasp host and they had only 5 unique compounds on their cuticle, but the beetles only shared 34 compounds with the German wasp (Table 1). Quantitatively, especially the beetle's methylalkane profile, which is generally considered to be important in nestmate recognition (van Zweden and d'Ettorre 2010), resembled that of *V. vulgaris* and not that of *V. germanica*. Our behavioural assays showed that common wasp host workers are also less aggressive towards the beetles than German wasp workers, suggesting that the chemical (CHC) mimicry is effective enough to reduce host aggression. We cannot exclude, however, that other appeasing or repelling substances may also play a role in preventing host attacks.

The similarity in CHC profiles between *M. paradoxus* and *V. vulgaris* raised the question if *M. paradoxus* produces host-specific hydrocarbons *de novo*, whether it acquires them through contact with the workers, or recycles the compounds from its prey. Our results of the isolation experiment (Figure 4) indicate that at least acquisition through contact with the adult workers is of minor importance in obtaining the host-like CHC profile, since isolated beetles showed very similar profiles to non-isolated beetles. Moreover, only female beetles appeared to be affected by the isolation, in the sense that their profiles plotted further away from the wasp hosts, showing that this was not universally true for all beetles but sex-specific (Figure 4). The isolation effect that we see here may also be confounded with age, since we kept the beetles in isolation alive for more than five days and we do not know the exact age of the beetles collected from natural colonies, although we assume that they reside inside the nest for maximum a couple of days (cf. Heitmans and Peeters 1996).

Hence, this change in CHC profile may well be linked to sexual maturity or fertility of the females. A striking example of the acquisition of host-specific compounds through contact with the host nest is found in the myrmecophilous beetle *Myrmecaphodius excavaticollis*, a parasite in *Solenopsis richteri* ant nests, where the beetles lost all host-specific cuticular compounds after an isolation period of two weeks, leaving a cuticular profile innate to the beetle (Vander Meer and Wojcik 1982). Similarly, *Varroa destructor* mites, which are parasites of the honey bee *Apis mellifera*, acquire pupa-specific methylalkanes within 3-9 hours of exposure to this host and can largely lose their profile within 18 hours of full isolation (Kather et al. 2015). The results of our isolation experiment with *M. paradoxus* appear to show the exact opposite in that the CHC profile remained principally unchanged after isolation from the wasp nest. This is more similar to, for example, parasitic bumble bees that have remained isolated in winter but still produce a host-specific odour profile (Martin et al. 2010).

These results may be explained when considering the beetle's life cycle, as the time that adult beetles spend inside the host nest is rather limited, and the observed apparent imperfect mimicry of host CHC profiles may be enough to exit the nest in adult state. Our observation that beetles were less attacked by workers from their own host colony (Figure 1) does indicate that there is some colony-specificity in their odour that helps to avoid being attacked, thereby implicating CHCs as likely candidate cues. Nonetheless, the beetles may very well also use other appeasing or repelling substances that lower received aggression, such as has been observed in several myrmecophilous parasites (d'Ettorre et al. 2000, Lenoir et al. 2001, Nash and Boomsma 2008).

Our results indicate that the CHC profile is, at least partially, recycled from the host larvae on which the beetles feed during their larval stage, since the CHC profiles of beetles varied according to the caste to which their prey larvae belonged (Table 2). One possibility is that the caste-specific CHC profiles were already present on the wasp larvae and the parasitic beetle larvae either directly obtained these hydrocarbons from the larva or indirectly from the cell walls. However, the finding that the beetle's odour profile remained virtually unchanged after several days of isolation (Figure 4) suggests an active form of mimicry, unlike the passive mimicry of the abovementioned *Varroa* mites (Kather et al. 2015). Alternatively, they could obtain

the building blocks in the right proportions to produce a caste-specific CHC profile in adult life. Similar results were reported in the myrmecophilous spider *Cosmophasis bitaeniata* that obtains its host-specific hydrocarbon profile through predation of ant larvae (Elgar and Allan 2004), although here too the exact mechanism is unknown. Such a system of CHC recycling is likely beneficial and selected for in parasites that feed on larvae, as they already resemble their hosts to a certain extent once they emerge from their cocoons, but is likely also partly explained by the fact that hydrocarbons are usually not assimilated by the digestive system, but instead are expressed on the cuticle of the individual (Guerrieri et al. 2009).

Lastly, both the beetles and queens show high relative proportions of linear alkanes compared to worker wasps (Figure 2; Table 1). Especially n-C_{29} seems to be overproduced by the beetles, which is one of three *n*-alkanes (n-C_{27}, n-C_{28}, and n-C_{29}) that have recently been identified as worker sterility-inducing queen pheromones (Van Oystaeyen et al. 2014), suggesting that this could be an additional strategy to avoid attacks by workers. Chemical mimicry of queens has, for example, also been reported in social parasites, such as *Apis mellifera capensis* workers that produce a series of queen pheromones allowing them to successfully infiltrate and reproduce in *Apis mellifera scutellata* colonies (Wossler 2002). It is still unclear, however, whether *M. paradoxus* produces high amounts of *V. vulgaris* queen pheromones to avoid aggressive behaviour by the workers, and requires further testing.

In conclusion, our results demonstrate that the parasitoid beetle *M. paradoxus* is chemically adapted to its typical host, the common wasp *V. vulgaris*, although the mimicry is not perfect. We show that host-specific hydrocarbons are not primarily acquired through contact with the adult host, but more likely through contact with the larval cell walls or through recycling hydrocarbons from the consumed larvae.

AUTHOR CONTRIBUTIONS

AVO, HH, WB and TW designed the experiments. AVO and HH collected wasp nests and carried out experiments. AVO, JSvZ, HH and FD analysed the data. AVO, JSvZ, and TW wrote the manuscript.

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